PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 16/00, 16/18, 16/44, C12Q 1/68, G01N 33/567

A1

(11) International Publication Number: **WO 99/50300**

(43) International Publication Date: 7 October 1999 (07.10.99)

(21) International Application Number: PCT/US99/06710

(22) International Filing Date: 29 March 1999 (29.03.99)

(30) Priority Data:

60/080,015	30 March 1998 (30.03.98)	US
60/095,383	5 August 1998 (05.08.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	60/080,015 (CIP)
Filed on	30 March 1998 (30.03.98)
US	60/095,383 (CIP)
Filed on	5 August 1998 (05.08.98)

(71) Applicant *(for all designated States except US)*: THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Center for Technology Transfer, Suite 300, 3700 Market Street, Philadelphia, PA 19104 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: TROJANOWSKI, John, Q. [US/US]; 2005 Pine Street, Philadelphia, PA 19103 (US). LEE, Virginia, M.-Y. [US/US]; 2005 Pine Street, Philadelphia, PA 19103 (US). IWATSUBO, Takeshi [JP/JP]; 4-39-2-401, Houkomagome, Bunkyo-ku, Tokyo 113 (JP).

(74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF IDENTIFYING, DIAGNOSING AND TREATING SYNUCLEIN POSITIVE NEURODEGENERATIVE DISORDERS

(57) Abstract

Methods of detecting synucleins to identify and diagnose synucleinopathies are provided. In addition, methods of treating such neurodegenerative disorders via modulation of production of selected synucleins and/or the formation of filamentous intracytoplasmic neuronal and glial inclusions from selected synucleins are described. A monoclonal antibody raised against Lewy bodies and specific for human α-synuclein is also provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHOD OF IDENTIFYING, DIAGNOSING AND TREATING SYNUCLEIN POSITIVE NEURODEGENERATIVE DISORDERS

Introduction

This invention was supported in part by funds from the U.S. government (NIH Grant No. AG09215) and the U.S. government may therefore have certain rights in the invention.

Background of the Invention

Parkinson's disease is the most common neurodegenerative disorder affecting brainstem extrapyramidal neurons of middle-aged individuals. Clinical features of Parkinson's disease are readily recognizable and include involuntary tremors, weakened muscles, a stooped posture, and eventually incapacitating rigidity and tremors. Although intellectual brain centers are not affected in early Parkinson's disease, dementia has become an increasingly recognized feature of advanced Parkinson's disease. The most regularly observed changes in brain tissue samples from patients suffering Parkinson's disease patients include degeneration of melanin-containing brainstem nerve cells, especially in the substantia nigra and the locus coeruleus, nerve cell loss with reactive gliosis in these same areas, and the presence of distinctive eosinophilic intracytoplasmic inclusions, or Lewy bodies in these nerve cells. Indeed, these spherical filamentous inclusions or Lewy bodies, which form in the perikarya of degenerating brainstem neurons, are considered the hallmark or diagnostic lesion of Parkinson's disease.

Lewy bodies also occur commonly in the brains of patients with the typical clinical and pathological features of Alzheimer's disease. Further, numerous cortical Lewy bodies are the defining brain lesions in another late-life neurodegenerative disorder known as dementia with Lewy bodies. Dementia with Lewy bodies is the second most frequent dementia after Alzheimer's disease.

Lewy bodies contain masses of 7-25 nm diameter filaments that appear similar to neurofilaments by electron microscopy. Neurofilament subunits and ubiquitin are implicated as major components of Lewy bodies in sporadic Lewy body diseases such as Parkinson's disease and dementia with Lewy bodies. However, the precise molecular composition of Lewy bodies is unclear, as is their role in the degeneration of neurons in Parkinson's disease and dementia with Lewy bodies as well as in cases of Alzheimer's disease with frequent Lewy bodies.

Synucleins are a family of soluble presynaptic proteins that are highly expressed in neurons. Identified members of the synuclein family include α-synuclein (also known as the non-amyloid component of plaques precursor protein, NACP) (Ueda, K. et al. 1993. *Proc. Natl Acad. Sci. USA*, 90:11282-11286), β-synuclein (also known as phosphoneuroprotein 14, PNP14) (Tobe, T. et al. 1992. *J. Neurochem.* 1992. 59:1624-1629; Nakajo, S. et al. 1993. *Eur. J. Biochem.* 217:1057-1063) and γ-synuclein (also known as breast cancer specific gene 1, BCSG1 or persyn) (Ji, H. et al. 1997. *Cancer Res.* 57:759-764; Ninkina, N.N. et al. 1998 *Hum. Mol. Genet.* 7:1417-1424). The three proteins although homologous, are encoded by three different genes mapped to chromosomes 4q21.3-q22 (α-synuclein) (Campion, D. et al. 1995. *Genomics* 26:254-257; Chen, X. et al. 1995 *Genomics* 26:425-427), 5q35 (β-synuclein) (Spillantini, M.G. et al.

1995. *Genomics* 27:379-381) and 10q23 (γ-synuclein) (Lavedan, C. et al. 1998. *Hum. Genet.* 103:106-112).

A synuclein protein was identified initially in *Torpedo* electroplaques and in the rat brain (Maroteaux, L. et al. 1988. *J. Neurosci.* 8:2804-2815; Maroteaux, L. and R.H. Scheller. 1991. *Mol. Brain. Res.* 11:335-343). A fragment of the 140 amino acid long human α-synuclein protein has been reported to be present in some amyloid plaques of brain tissue samples from patients with Alzheimer's disease. Subsequent studies suggest that this peptide may contribute to the fibrillogenesis of the β-amyloid peptide in senile plaques (Goedert, M. 1997. *Nature* 388:232-233; Heintz, N. and H. Zoghbi. 1997. *Nature Genet.* 16:325-327). The peptide fragment of human α-synuclein has been designated the non-amyloid β component (NAC) of amyloid plaques while the precursor protein of this peptide has been designated NACP (Goedert, M. 1997. *Nature* 388:232-233).

Recent studies have shown that α-synuclein, a 140-amino acid protein localized to presynaptic terminals, harbors an *Ala* to *Thr* substitution at position 53 (A53T) due to a mis-sense mutation of the α-synuclein gene in four pedigrees with autosomal-dominantly inherited familial Parkinson's disease (Polymeropoulos, M.H. et al. 1997. *Science* 276:2045-2047). Studies have also shown that α-synuclein, but not β-synuclein, is a component of Lewy bodies in sporadic Lewy body diseases including Parkinson's disease and dementia with Lewy bodies (Spillantini, M.G. et al. 1997. *Nature* 388:839-840; Takeda, A. et al. 1998. *Am. J. Pathol.* 152:367-372). In these studies, substantia nigra sections from Parkinson's disease and dementia with Lewy bodies, and cingulate cortex sections from dementia with Lewy bodies and dementia with Lewy bodies with Alzheimer's disease were incubated with antibodies raised

against synthetic peptides corresponding to residues 11-34 or 116-131 of human α-synuclein.

A monoclonal antibody raised against Lewy bodies which is specific for human α-synuclein has now been identified. This monoclonal antibody has now been used to immunoassay biological samples to detect neurodegenerative disorders characterized by filamentous intracytoplasmic neuronal and glial inclusions, also referred to herein as filamentous aggregates, comprising α-synuclein. For example, using this antibody, it has now been found that α-synuclein is present in the filaments which aggregate into Lewy bodies and Lewy neurites in neurons of Parkinson's disease, Alzheimer's disease and dementia with Lewy bodies, the tubules and/or filaments that aggregate in the glial cell inclusions that are diagnostic of Multiple System Atrophy, and in the filamentous aggregates of Hallervorden-Spatz disease. Further, novel neuritic pathologies have now been identified with antibodies to β- and γ-synuclein in the hippocampus of brains from patients suffering from Parkinson's disease and dementia with Lewy bodies, but not in brains from control patients, thereby implicating all three known synuclein isoforms in the pathogenesis of neurodegenerative diseases. Neurodegenerative diseases or disorders characterized by these synuclein positive filamentous aggregates are referred to herein collectively as "synucleinopathies."

Summary of the Invention

An object of the present invention is to identify a neurodegenerative disorder as a synucleinopathy which comprises detecting selected synucleins in filamentous aggregates characteristic of the neurodegenerative disorder.

Another object of the present invention is to provide a method of diagnosing synucleinopathies in patients which

comprises detecting selected synucleins in a biological sample obtained from the patient.

Yet another object of the present invention is to provide a method of treating synucleinopathies in patients suffering from neurodegenerative disorders which comprises modulating production of selected synucleins and/or the formation of filamentous intracytoplasmic neuronal and glial inclusions from selected synucleins in these patients. Yet another object of the present invention is to provide a monoclonal antibody raised against Lewy bodies which is specific for human α-synuclein.

Yet another object of the present invention is to provide a method of detecting α-synuclein in a biological sample with a monoclonal antibody raised against Lewy bodies which is specific for human α-synuclein.

Detailed Description of the Invention

It has now been demonstrated using a monoclonal antibody specific for human α-synuclein that α-synuclein is an integral component of the filaments which aggregate into both brainstem and cortical Lewy bodies and Lewy neurites as well as the tubules and/or filaments that aggregate in glial cell inclusions. It is believed that mechanisms leading to the selective incorporation of α-synuclein into these filamentous intracytoplasmic neuronal and glial inclusions from α-synuclein, also referred to herein as filamentous aggregates, involve alterations in the metabolism of this molecule that render it insoluble and prone to aggregation. Thus, wild-type α-synuclein is believed to play a role in the pathogenesis of various neurodegenerative disorders including, but not limited to, Parkinson's disease, dementia with Lewy bodies, familial Alzheimer's disease, Multiple System Atrophy and Hallervorden-Spatz disease, while mutations in the α-synuclein gene produce a mutant form of the protein which

augments this process.

In addition, it has now been found that dentate, hilar and hippocampal CA2/3 regions of brains from patients suffering from Parkinson's disease and dementia with Lewy bodies contain abnormal axon terminals immunoreactive not only for α-synuclein, but also β-synuclein. Further, γ-synuclein positive axonal spheroids have now been found to be present in the dentate molecular layer. Antibodies to several synaptic proteins, (synapsin, synaptophysin, synaptobrevin) also label this pathology, none of which was seen in control brains. It is believed that synucleins, including, but not limited to, α-, β- and γ-synuclein, play a role in the onset/progression of neurodegenerative disorders including, but not limited to, Parkinson's disease, dementia with Lewy bodies, familial Alzheimer's disease, Multiple System Atrophy and Hallervorden-Spatz disease. Neurodegenerative disorders with synuclein positive filamentous aggregates are referred to collectively herein as "synucleinopathies".

The present invention relates to antibodies specific for selected synucleins, including but not limited to α-, β- and γ-synuclein, and methods of using such antibodies to identify neurodegenerative diseases as synucleinopathies, to detect selected synucleins in biological samples to diagnose these diseases, and to study the role of selected synucleins in synucleinopathies and to identify agents which modulate production of selected synucleins and/or the formation of filamentous intracytoplasmic neuronal and glial inclusions from synucleins in these disorders. For the purposes of this invention, by "selected synucleins" it is meant to include any synuclein. Specific examples of human synucleins include α-synuclein, β-synuclein and γ-synuclein. However other synucleins may also be present in synucleinopathies and would be useful in identifying, diagnosing and treating such neurodegenerative disorders.

The presence of α-synuclein in Lewy bodies was first determined using antiserum to synthetic synuclein peptides or purified synuclein proteins. Antiserum 77 was raised against purified bovine β-synuclein. This antiserum recognized both human α- and β-synucleins. Antiserum 259 was raised against a synthetic peptide corresponding to residues 104 to 119 of rat α-synuclein. This antisera recognized human α-synuclein but not human β-synuclein. A third antiserum was raised to a synthetic peptide identical to residues 91 to 105 of rat β-synuclein. This antiserum recognized only purified human β-synuclein. The ability of these antisera to stain Lewy bodies was examined. No immunolabeling of Lewy bodies was observed with the β-synuclein-specific antiserum 253. In contrast, antiserum 259 and 77 specifically and intensely labeled Lewy bodies within the cytoplasm of dopaminergic substantia nigra neurons, neurons specifically affected in Parkinson's disease and dementia with Lewy bodies. The α-synuclein immunoreactivity in brainstem Lewy bodies was more intense than that obtained using anti-ubiquitin antibodies. Absorption of antiserum 259 with its peptide immunogen completely abolished Lewy body staining. Antisera 259 and 77 also immunolabeled cortical Lewy bodies as strongly and frequently as anti-ubiquitin antibodies. Tau-positive neurofibrillary tangles and neuropil threads in some of these sections were never labeled with the anti-synuclein antibodies in double-label confocal microscopy studies. However, α-synuclein-positive neurites, which were distinct from tau-positive neuropil threads, were found in regions of dementia with Lewy body cortex showing severe neuronal loss and numerous Lewy bodies. These abnormal processes clearly resembled CA2/3 neurites in the hippocampus of dementia with Lewy bodies patients (Dickson, D.W. et al. 1991. *Neurology* 41:1409). These neurites also were strongly α-synuclein positive. These α-synuclein-labeled

neuritic lesions were present in the brains of all of the Parkinson's disease and dementia with Lewy bodies cases studied. These inclusions also were strongly immunolabeled in smears of unfixed, isolated Lewy bodies with antisera 259 and 77, as well as with anti-ubiquitin antibodies.

To characterize the ultrastructural localization of α-synuclein immunoreactivity in Lewy bodies, immunoelectron microscopy was performed with antiserum 259. The peripheral portions of brainstem Lewy bodies were immunolabeled with antiserum 259, which contained primarily decorated filaments and associated granular material in the inclusions. Unfixed cortical Lewy bodies were also examined by an immunogold immuno-EM technique, and the gold particles localized to amorphous material associated with the filaments in Lewy bodies. These results confirmed that α-synuclein was localized in Lewy bodies.

Lewy bodies were then purified from brain tissue samples obtained at autopsy from patients suffering from dementia with Lewy bodies. Monoclonal antibodies to Lewy body immunogens were then generated using 3 x 10^6 purified Lewy bodies. Hybridoma supernatants were screened and specificity of selected MAbs was characterized by immunoblot analyses using heat-stable, high-salt extractable fraction from homogenates of human brain samples taken at autopsy. An α-synuclein-specific MAb, clone LB509, was identified that was capable of intensely staining numerous intraneuronal Lewy bodies throughout substantia nigra and neocortex tissue sections of postmortem brains from seven sporadic Parkinson's disease and nine dementia with Lewy bodies patients.

MAb LB509 stained Lewy bodies equally well in brains fixed with 70% ethanol or 10% formalin, as well as unfixed smears of the isolated Lewy bodies. MAb LB509 specifically reacted with an approximately 18-kDa polypeptide in the heat-stable soluble fraction of human cingulate gyrus on

Western blots shown to be α-synuclein by demonstrating that LB509 specifically reacted with purified human α-synuclein, which migrated at approximately 18 kDa, but not with β-synuclein which ran with a slightly slower mobility.

To biochemically characterize the α-synuclein in Lewy bodies, Western blot analyses were performed on more highly purified, formic-acid-treated Lewy bodies isolated from dementia with Lewy body cortex. MAb LB509 labeled an immunoband with a molecular weight similar to normal human α-synuclein, as well as 14 to 16 kDa immunobands, consistent with partially truncated forms of α-synuclein. Several other immunostained bands migrating at 25 to 50 kDa, as well as higher molecular weight α-synuclein-immunoreactive aggregates and labeled material that did not enter the stacking gel, were also detected by MAb LB509. Similar protein bands were detected in Western blots of isolated Lewy bodies probed with antiserum 259.

α-synuclein immunoreactivity was consistently enhanced in sections pretreated with formic acid in accordance with procedures described by Takeda A. et al. 1998. *Lab. Invest.* 78:1169-1177. In addition, many hilar neurons in both Parkinson's disease and dementia with Lewy bodies cases were surrounded by accumulations of α-synuclein positive punctate or vesicular profiles. Although some α-synuclein positive vesicular profiles were present around the perikarya of hilar neurons in brains of control and Alzheimer's disease cases (Table 1), the marked abundance and variable size of those in the brains of Parkinson's Disease and dementia with Lewy bodies cases indicates that they reflect pathological aggregates of α-synuclein in axon terminals of mossy fiber projections to hilar neurons. The vesicular profiles were not seen in the multiple system atrophy or Pick's disease cases examined (Table 1). The abundance of these vesicles appears to parallel that of α-synuclein positive Lewy bodies in

entorhinal cortex and Lewy neurites in the CA2/3 regions of the Parkinson's Disease and dementia with Lewy bodies cases. Tissue sections of these vesicles pretreated with formic acid were also β-synuclein positive but γ-synuclein negative. However, axonal spheroid-like lesions were identified in the stratum moleculare of the dentate gyrus of Parkinson's disease and dementia with Lewy bodies cases with antibodies to γ-synuclein, but not with anti-α-synuclein or anti-β-synuclein antibodies. These axonal spheroids were most apparent following pretreatment with formic acid, otherwise they were difficult to distinguish from normal neuropil staining. While similar γ-synuclein positive spheroids were seen in the CA1 region, they were most abundant in the dentate molecular layer. Immunoreactivity for several different synaptic proteins (e.g. synapsin, synaptophysin and synaptobrevin) in the hilar vesicular profiles was also observed, thus indicating pathologically altered axon terminals or synapses. The γ-synuclein positive spheroids in the dentate molecular layer are believed to be a site of more proximal pathology, and they were positive only with antibodies against synaptobrevin and not to the other synaptic proteins. β-synuclein and γ-synuclein pathology was not seen in normal cases or in cases of Alzheimer's Disease, Multiple System Atrophy or Pick's disease (Table 1).

TABLE 1: Synuclein-immunoreactive hippocampal pathology in neurodegenerative disorders

Disease	Total Cases	α-synuclein	β-synuclein	γ-synuclein
Normal	5	2/5	0/5	0/5
Parkinson's Disease	5	5/5	5/5	5/5

Dementia with Lewy bodies	5	5/5	5/5	5/5
Alzheimer's Disease	5	2/5	0/5	0/5
Pick's disease	2	2/2	0/2	0/2
Multiple system atrophy	2	2/2	0/2	0/2

Thus, α-, β- and γ-synuclein pathology was consistently seen in all Parkinson's Disease and dementia with Lewy bodies cases. In normal and Alzheimer's Disease cases, no Lewy bodies or Lewy neurites were seen, however, a few hilar neurons demonstrated α-synuclein immunoreactivity suggestive of degenerating axon terminals. This immunoreactivity was similar, though substantially reduced when compared to the Lewy body disorders. No β- or γ-synuclein pathology was noted.

The Pick's disease cases demonstrated Pick bodies immunoreactive for α-synuclein in the dentate and parahippocampal gyrus, however, no degenerating axon terminals were seen. No β- or γ-synuclein pathology was noted.

In the multiple system atrophy cases, multiple glial cytoplasmic inclusions immunoreactive for α-synuclein were noted in the white matter of the hippocampus, however, few degenerating axon terminals were noted. No β- or γ-synuclein pathology was noted.

Pre-embedding methods revealed that α-synuclein was localized to several locations in the hippocampal hilus. There were various neuronal processes (with and without myelin sheaths and the terminal complexes of mossy fiber rosettes) immunoreactive for α-synuclein. The rosettes appeared to be predominantly surrounding the hilar neurons as identified by light microscopy. Although the

ultrastructural preservation of the tissue did not allow exact localization of the DAB chromagen, the immunoreactivity appeared to be greatest on the vesicle-like structures densely accumulated within axon terminals.

In additional experiments, α-synuclein positive Lewy bodies were also identified in patients suffering from familial Alzheimer's disease. Tissue samples used in these experiments came from 58 familial Alzheimer's disease patients with mutations in the Presenilin-1 gene, 9 familial Alzheimer's disease patients with mutations in the amyloid precursor protein gene, and 7 familial Alzheimer's disease patients with a mutation in the Presenilin-2 gene. The brain samples that were available for study came from the following regions: substantia nigra (N=15), locus ceruleus (N=12), amygdala (N=19), periamygdaloid enterohinal cortex (N=18), parahippocampal gyrus (at the level of the lateral geniculate nucleus, N=18), cingulate gyrus (N=25), middle frontal cortex (N=66) and cerebellum (N=11). An abundance of α-synuclein positive Lewy bodies was observed immunohistochemically in the amygdala of 12 out of 19 available samples. α-synuclein positive Lewy bodies also were identified in the adjacent periamygdaloid cortex in 8 of the 18 samples examined, the parahippocampal gyrus in 4 of the 18 samples examined, the cingulate gyrus in 6 of the 25 samples examined and areas 8 and 9 of the frontal lobe in 9 out of 66 samples examined. Fewer Lewy bodies were stained by the α-synuclein antibodies in the substantia nigra (2 out of 15 of the samples) and in the locus ceruleus (1 of the 12 samples examined). No α-synuclein aggregates were seen in the cerebellum of any of the samples from patients with familial Alzheimer's disease.

The α-synuclein specific MAb LB509 also intensely stained glial cytoplasmic inclusions throughout the white matter in 9 of 13 Multiple System Atrophy cases. Multiple

System Atrophy is a syndrome complex that includes olivopontocerebellar atrophy, striatonigral degeneration and Shy-Drager disease. The abundance of α-synuclein positive glial cytoplasmic inclusions varied from region to region in the brains of different Multiple System Atrophy types. For example, in all Shy-Drager disease variants, there were numerous α-synuclein immunoreactive glial cytoplasmic inclusions in white matter as well as intraneuronal inclusions in grey matter of almost all brain regions examined. Occasional intranuclear α-synuclein positive inclusions also were seen.

Antibodies to α-synuclein, but not β- or γ-synuclein also stained intraneuronal inclusions in grey matter of cingulate cortex and putamen as well as glial cytoplasmic inclusions and axonal swellings in the corresponding white matter of the brain from a patient with the rare movement disorder, Hallervorden-Spatz disease.

Accordingly, a monoclonal antibody of the present invention, which is specific for α-synuclein is useful in immunoassays for the detection of α-synuclein in biological samples. Increased or elevated levels of α-synuclein in the biological sample as compared to α-synuclein levels in biological samples from normal healthy volunteers can be used in diagnosing synucleinopathies including, but not limited to, dementia with Lewy bodies, Parkinson's disease, familial Alzheimer's disease, Multiple System Atrophy and Hallervorden-Spatz disease. Additional monoclonal or polyclonal antibodies against selected synucleins, such as α-synuclein, β-synuclein or γ-synuclein, can also be used in the detection of selected synucleins, wherein increases of elevated levels of selected synucleins are diagnostic for synucleinopathies.

Antibody-based methods for detection of proteins in biological samples including, but not limited to, cerebrospinal fluid (CSF), blood, plasma and serum are well

known to those skilled in the art. Examples of such assays include, but are not limited to, enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs). In these assays, a solid phase support is coated with an antibody specific for a selected synuclein, preferably either α-, β- or γ-synuclein. A biological sample, preferably blood, serum, plasma or CSF, obtained from a patient suspected of suffering from a synucleinopathy is then exposed directly to the solid phase support to which the anti-synuclein specific first antibody has been attached so that any of the selected synuclein in the sample is retained on the support. The support is then washed to remove excess unbound material when appropriate, as is known in the art. Synuclein bound to the solid phase support is then detected, preferably via a second detectably labeled antibody specific for the selected synuclein, so that the presence and/or quantity of the selected synuclein in the biological sample can be determined. Examples of detectable labels include radioisotopes or enzymes such as acetylcholinesterase, alkaline phosphatase, α-glycerophosphate dehydrogenase, asparaginase, β-galactosidase, β-V-steroid isomerase, catalase, glucoamylase, glucose oxidase, glucose-6-phosphate dehydrogenase, horse radish peroxidase, malate dehydrogenase, ribonuclease, staphylococcal nuclease, triose phosphateisomerase, urease and yeast alcohol dehydrogenase.

As will be obvious to those of skill in the art upon this disclosure, alternative methods for detecting elevated levels of a selected synuclein in a biological sample such as nucleic acid methods can also be used in the present invention. Examples of nucleic acid methods include, but are not limited to polymerase chain reaction (PCR), ligase chain reaction (LCR) and nucleic acid based amplification (NASABA). Reverse transcriptase PCR (RT-PCR) also provides

a powerful tool useful in the detection of the presence of a specific mRNA population in a complex mixture of thousands of other mRNA species. Additional methods obvious to those of skill in the art upon this disclosure for determining synuclein levels in biological samples can also be used.

These methods of detecting selected synucleins, preferably α-, β- or γ-synuclein, are also useful in identifying additional neurodegenerative disorders as synucleinopathies. Further, such methods, and in particular antibody-based methods, are useful in studying the role of selected synucleins in the pathogenesis of these diseases and in the development of agents which modulate production of synucleins in these diseases. It is believed that agents which inhibit production of synucleins will be useful in inhibiting formation of the filamentous aggregates which are characteristic of synucleinopathies. Such agents can be administered to a patient to inhibit production of a synuclein and/or the formation of the filamentous intracytoplasmic neuronal and glial inclusions from synucleins.

The present invention is further described by the following examples. These examples are provided solely to illustrate the invention by reference to specific embodiments. These exemplifications, while illustrating certain aspects of the invention, do not portray the limitations or circumscribe the scope of the disclosed invention.

EXAMPLES

Example 1: Monoclonal Antibodies

Monoclonal antibodies to Lewy body immunogens were generated using approximately 3 x 10^6 Lewy bodies purified from dementia with Lewy bodies cortices. Hybridoma supernatants were screened by immunostaining unfixed smears

of post-sucrose fractions of isolated Lewy bodies as described by Iwatsuba, T. et al. 1998. *Am. J. Pathol.* 148:1517-1529, except that immunogens were disrupted by repeated ultrasonication in 0.5 ml of 50 mmol/L Tris-buffered saline (pH 7.6) without denaturation by formic acid. The specificity of the monoclonal antibodies was further characterized by immunoblot analyses using heat-stable, high-salt extractable fraction from homogenates of human brain. For the production of polyclonal antibodies to α- and β-synuclein, synthetic synuclein peptides were used as immunogens. These peptides included nonhomologous amino acid sequences in α- and β-synuclein that were well conserved in the rat and human forms of these proteins (93.8% for α-synuclein and 100% for β-synuclein). The amino termini of each of the peptides were conjugated to keyhole limpet hemocyanin via a cysteine residue for use as immunogens in rabbits to generate antisera 253 and 259 whereas antiserum 77 was raised against purified bovine PNP14 (β-synuclein).

Additional monoclonal antibodies used in immunohistochemical assays included Syn207 targeted against β-synuclein (Tu, P.H. et al. 1998. *Ann. Neurol.* 44;415-422), Antisera20 targeted against γ-synuclein (Tu, P.H. et al. 1998. *Ann. Neurol.* 44;415-422), 1510 targeted against ubiquitin (Chemicon, Temecula, CA), SY38 targeted against synaptophysin (Boehringer-Mannheim, Indianapolis, IN), anti-synapsin I (Molecular Probes, Eugene, OR) and MAB335 targeted against synaptobrevin (Boehringer-Mannheim).

Example 2: Case Materials

Autopsy proven cases of 5 patients with Parkinson's Disease, 5 patients with dementia with Lewy bodies and 5 normal controls were compared. In addition 5 cases of Alzheimer's Disease, 2 cases of multiple system atrophy and 2 cases of Pick's disease were examined. Material was

obtained from the brain bank at the Center for Neurodegenerative Disease Research (CNDR). Brains were fixed in 10% formalin, Bouin's solution or 70% ethanol/150 mM NaCl and embedded in paraffin. Serial sections were cut at 6 μm thickness.

Example 3: Immunoblot Analyses

Purified human α- and β-synuclein proteins (50 ng for MAb LB509 and antisera 77 and 259 and 100 ng for antiserum 253) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 13% gel, transferred to a nitrocellulose membrane, incubated with mouse MAb LB509 (culture supernatant, 1:10) or rabbit antisera 77 (1:2000), 259 (1:1500), and 253 (1:1500), respectively, and visualized by enhanced chemiluminescence. The specificity of both the MAbs and polyclonal antibodies were further assessed using a heat-stable soluble fraction (100,000 x g) of human cingulate gyrus homogenized in 1.5 volumes of a buffer containing 0.75 mol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol, 0.1 mol/L MES, pH 7.0, and a cocktail of protease inhibitors as well as purified human α- and β-synucleins separately recovered on a DEAE-cellulose column. For immunoblot analysis of purified Lewy bodies, approximately 200,000 purified Lewy bodies were solubilized in 70% formic acid, desiccated, resolubilized in SDS sample buffer, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with LB509.

Example 4: Immunocytochemistry

Sections were de-paraffinized in xylene and rehydrated in descending concentrations of ethanol. One half of the sections were treated with 88% formic acid for 1 minute and washed in distilled water for 5 minutes. After treatment with methanol/H_2O_2, sections were pre-incubated in Tris-buffered saline (TBS), pH 7.4. Sections

were blocked in TBS/0.08% Triton X-100/2% horse serum and incubated with antibodies against α-synuclein (LB509 and Syn208 (Tu, P.H. et al.1998. Ann. Neurol. 44:415-422), β-synuclein (Syn207), γ-synuclein (Antisera20), ubiquitin (1510, Chemicon, Temecula, CA), synaptophysin (SY38, Boehringer-Mannheim, Indianapolis, IN), synapsin I (Molecular Probes, Eugene, OR) and synaptobrevin (MAB335, Boehringer-Mannheim) overnight at 4°C. Sections were developed using the avidin-biotin method (ABC, Vector Labs, Burlingame, CA) with DAB as the chromogen. Images were captured with a Nikon X-100 microscope and imported into Northern Exposure (Empix Inc).

Example 5: Electron Microscopy

Electron microscopy was performed on the dementia with Lewy body brain. Specifically, a piece of hippocampus was removed from frozen sections of 3 DLB cases. The tissue block was fixed immediately in phosphate buffered (pH 7.4) fixative containing 0.1% glutaraldehyde and 4% paraformaldehyde overnight. The blocks were then sliced at 50 μm thickness. To identify the immunoreactive structures seen at the light microscopic level, pre-embedding immunoelectron microscopy with antibodies against α-synuclein and γ-synuclein. These sections were washed well in phosphate buffered saline (PBS), blocked with 2% horse serum to eliminate non-specific immunoreaction and incubated with primary antibody overnight at 4°C. The following day, the sections were washed well with PBS, incubated with biotinylated anti-mouse IgG (Vector Labs) for one hour and washed again. The sections were then developed using the ABC method (Vector Labs) with DAB as a chromogen. Sections were dehydrated and embedded in epoxy resin. Ultrathin sections were prepared and viewed.

To identify the precise localization of the immunostaining, post-embedding immunoelectron microscopy

was performed as follows. Sections were washed with PBS, dehydrated and embedded with LR white (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were cut and processed for immunohistochemistry. The sections were first blocked with horse serum and then incubated with primary antibodies overnight at 4°C. The sections were washed in PBS and incubated with anti-mouse IgG conjugated with 10 nm gold particles (Electron Microscopy Sciences). After light staining with uranium acetate (Electron Microscopy Sciences), localization of immunogold particles was examined.

What is claimed is:

1. A method of identifying a selected neurodegenerative disorder as a synucleinopathy which comprises:

(a) obtaining a biological sample from a patient diagnosed with a selected neurodegenerative disorder;

(b) measuring a level of selected synuclein in the biological sample; and

(c) comparing the measured level of selected synuclein in the biological sample with selected synuclein levels in biological samples from normal healthy volunteers wherein elevated levels of the selected synuclein are indicative of the neurodegenerative disorder being a synucleinopathy.

2. The method of claim 1 wherein the level of selected synuclein in the biological sample is measured via an antibody-based assay.

3. A method for diagnosing a synucleinopathy in a patient comprising:

(a) obtaining a biological sample from a patient;

(b) measuring a level of a selected synuclein in the biological sample; and

(c) comparing the measured level of selected synuclein in the biological sample with selected synuclein levels in biological samples from normal healthy volunteers wherein elevated levels of selected synuclein are indicative of a synucleinopathy.

4. The method of claim 3 wherein the level of selected synuclein in the biological sample is measured via an antibody-based assay.

5. A method of treating a neurodegenerative disorder identified as a synucleinopathy in a patient comprising administering to a patient an agent which inhibits production of a selected synuclein.

6. A method of treating a neurodegenerative disorder identified as an synucleinopathy in a patient comprising administering to a patient an agent which inhibits formation of filamentous intracytoplasmic neuronal and glial inclusions from synucleins.

7. A monoclonal antibody raised against Lewy bodies which is specific for human α-synuclein.

8. A method of detecting α-synuclein in a biological sample comprising:

(a) exposing a biological sample to a solid phase support coated with a monoclonal antibody of claim 7 so that any α-synuclein in the biological sample is immobilized to the solid phase support; and

(b) detecting immobilized α-synuclein on the solid phase support.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06710

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 16/00, 16/18, 16/44, C12Q 1/68, G01N 33/567
US CL :530/387.1, 388.1, 388.85, 435/6, 91.2, 436/503

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.1, 388.1, 388.85, 435/6, 91.2, 436/503

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Author search

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

File Bioscience; Medline, CAPlus, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KRUGER et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat. Gen., February 1998, Vol. 18, No. 2., pages 106-108, see entire document.	1
X	SPILLANTI et al. Alpha-synuclein in Lewy Bodies. Nature. 28 August 1997, Vol. 388, pages 839-840, see entire document.	1
L	IRIZARRY et al. Characterization of the precursor protein of the non-abeta component of senile plaques (NACP) in the human central nervous system. J. of Neuropath & Exp Neurol. August 1996, Vol. 55, No. 8, pages 889-890, particularly discussion relevant with respect to Alzheimer's.	1

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 JUNE 1999	Date of mailing of the international search report 05 AUG 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SHARON L. TURNER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06710

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAKABASHI et al. NACP, a presynaptic protein, immunoreactivity in Lewy bodies in Parkinson's disease. Neurosci Ltrs. 1997, Vol. 239, pages 45-48, particularly page 47 column 1.	1
X	NUSSBAUM, R.L. et al. Genetics of Parkinson's disease. Hum Mol. Genet. 1997, Vol. 6, No. 10, pages 1687-1691, see entire document.	1
X,P	WO 98/59050 A1 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES) 30 December 1998, see entire document.	1
X,P	WAKABAYASHI et al. Accumulation of alpha-synuclein/NACP is a cytopathological feature common to Lewy body disease and multiple system atrophy. Acta Neuropath., 1998, Vol. 96, pages 445-452, see entire document.	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06710

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06710

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim 1, drawn to a method of identifying a neurodegenerative disorder as a synucleinopathy.
Group II, claim 2, drawn to a method of identifying a neurodegenerative disorder as a synucleinopathy in an antibody-based assay.
Group III, claim 3, drawn to a method of diagnosing a synucleinopathy.
Group IV, claim 4, drawn to a method of diagnosing a synucleinopathy in an antibody-based assay.
Group V, claim 5, drawn to a method of treating a synucleinopathy by administering an agent which inhibits production of a selected synuclein.
Group VI, claim 6, drawn to a method of treating a synucleinopathy by administering an agent which inhibits formation of neuronal and glial inclusions from synucleins.
Group VII, claim 7, drawn to a monoclonal antibody raised against Lewy bodies specific for human alpha-synuclein.
Group VIII, claim 8, drawn to a method of detecting a alpha-synuclein in a biological sample using a monoclonal antibody and immobilized alpha-synuclein on a solid phase support.

The inventions listed as Groups I-VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I, the first appearing method of identifying a synucleinopathy using the first appearing special technical features of a biological sample and synuclein measurement. Group I, the first appearing method and special technical features, anticipated by Kruger et al, Nature Genetics, Vol 18, No 2, 02/11/98, pages 106-108, Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease, which teaches from a biological sample, the detection of a level of a selected synuclein and comparison with diseased and normal samples. Group II, the second method of using the second special technical feature of an antibody, anticipated by Spillantini MG et al, Nature, Vol 388, 8/28/97, pages 839-840, Alpha-synuclein in Lewy bodies, which teaches from a biological sample, the detection of a level of a selected synuclein by antibody assay and comparison with diseased and nondiseased samples. Group III, the third method of using the first appearing special technical features. Group IV, the fourth method of using the first and second special technical features. Group V, the fifth method of using the third appearing special technical feature. Group VI, the sixth method of using the fourth appearing special technical feature. Group VII, the seventh method of using the fifth appearing special technical feature. Group VIII, the eigth method of using the eighth special technical feature.